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Electroencephalographic characterisation of pentylenetetrazole-induced seizures in mice lacking the α4 subunit of the neuronal nicotinic receptor

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Abstract

Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) is associated in some kindreds with mutations in the genes encoding the α4 or β2 subunits of the neuronal nicotinic acetylcholine receptor (nAChR). Functional characterisation of the described ADNFLE mutations in oocyte preparations has produced conflicting results, with some studies suggesting hypofunction but others showing increased ligand sensitivity or delayed desensitisation. Knockout mice were studied to investigate extreme hypofunction of α4 nAChRs in vivo. Mutant (Mt) and control mice underwent epidural electroencephalographic (EEG) recording for 2 h in the untreated state and for 1 h following administration of the γ-amino butyric acid (GABA) antagonist, pentylenetetrazole (PTZ, 80 mg/kg). No spontaneous seizures occurred and no EEG differences were observed between the genotypes in drug naïve mice. Following PTZ, however, Mt mice showed markedly increased mortality compared to controls (85 vs 30%, $P < 0.001$). Mts also had a greater number of generalised clonic seizures in the first 40 min following injection. In the same period, the EEGs of Mt mice showed an excess of spikes ($P = 0.033$), multi-spike complexes ($P = 0.002$) and continuous fast activity ($P = 0.017$) compared to controls. These findings demonstrate that intact α4 nAChR subunits provide significant in vivo protection against the proconvulsant effects of GABA antagonism.

Keywords: Electroencephalograph; Seizure; Epilepsy; Animal models; Pentylenetetrazole; Nicotinic

1. Introduction

Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) is an inherited disorder in which seizures occur during sleep. Five mutations have been identified in ADNFLE kindreds to date and all involve the α4 or β2 subunit of the neuronal nicotinic acetylcholine receptor (nAChR) (Steinlein et al., 1995, 1997; Hirose et al., 1999; Phillips et al., 2001; De Fusco et al., 2000). Neuronal AChRs are ligand-gated ion channels that, once activated, are permeable to sodium and calcium and cause cellular depolarisation. They are pentamers variously composed of α subunits (α2–α10) and β subunits (β2–β4) (Le Novere and Changeux, 1995; Elgoyhen et al., 2001). The most common configuration contains α4 and β2 subunits, while the α7 homooligomer also plays an important role (Gotti et al., 1997).

Activation of presynaptic nAChRs is known to facilitate the release of several neurotransmitters including γ-amino butyric acid (GABA), glutamate, dopamine and acetylcholine (ACh) itself (Wonnacott, 1997). Studies of GABAergic facilitation have primarily implicated α4/β2 nAChRs (Lena and Changeux, 1997; Lu et al., 1998). Glutamate facilitation appears to involve α7 nAChRs in many brain regions (Radcliffe and Dani, 1998; MacDermott et al., 1999) but may also involve α4/β2 nAChRs (Vidal and Changeux, 1993; Gil et al., 1997; Gioanni

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et al., 1999). The composition of nAChRs modulating dopamine release is uncertain; contributions from α3, α4, β2 and β4 subunits have been proposed (Grady et al., 2001; Wonnacott, 1997; MacDermott et al., 1999).

Postsynaptic nAChRs have also been demonstrated in many regions and application of nicotinic agonists in vitro can elicit cellular depolarisation and action potentials in nAChR-positive neurons (Xiang et al., 1998; Porter et al., 1999; Alkondon et al., 2000). There is also in vitro evidence implicating nAChRs in fast synaptic transmission in the hippocampus (Hefft et al., 1998; Frazier et al., 1998). Because the role of nAChRs in normal neurotransmission in vivo remains uncertain, however, aetiological hypotheses about ADNFLE have concentrated on the neuromodulatory role of nAChRs (Bertrand and Changeux, 1999). A defect in the usual α4/β2 mediated facilitation of GABA release would provide a plausible basis for epilepsy. This hypothesis would be strengthened if the described ADNFLE mutations were associated with hypofunction of α4/β2 nAChRs and if hypofunction of these receptors created a predisposition to seizures.

Functional characterisation of the ADNFLE mutations in vitro has produced an inconsistent picture. The described α4 mutations affect the second transmembrane domain of the α4 subunit (Steinlein et al., 1995, 1997; Hirose et al., 1999), while the described β2 mutations affect the corresponding domain of the β2 subunit (De Fusco et al., 2000; Phillips et al., 2001). Characterisation of the α4 S248F mutation in oocytes showed a significant decrease in ACh-induced currents and a shift in the dose–response curve indicating lower sensitivity (Steinlein et al., 1995; Kuryatov et al., 1997), whereas oocyte studies of the α4 259insL mutation revealed an increase in ACh affinity without significant changes in the total channel current (Steinlein et al., 1997; Bertrand et al., 1998). In both mutations, the ACh-evoked calcium current was reduced, which may be an important unifying observation given that calcium ions are implicated as second messengers in the neuromodulatory role of nAChRs (Lena and Changeux, 1997; Bertrand et al., 1998). However, while the above studies suggest receptor hypofunction, Figl et al. (1998) reported use-dependent potentiation of the nicotinic ACh response with these two mutations. Functional studies of the third known α4 mutation (S252L) suggest receptor hypofunction with similar affinity but faster desensitisation compared to wild type (Wt) receptors (Matsushima et al., 2002). Finally, characterisation of the described β2 mutations showed increased affinity for ACh, in the case of β2V287M, and delayed desensitisation, in the case of β2V287L, leading to increased current flow in oocyte preparations (De Fusco et al., 2000; Phillips et al., 2001).

In contrast to this divergent in vitro characterisation of the mutations, kindreds with ADNFLE have a broadly consistent phenotype (De Fusco et al., 2000; Phillips et al., 2001). This suggests that more complex models are required to determine the functional consequences of nAChR dysfunction in vivo.

To investigate the effect of extreme hypofunction of α4/β2 nAChRs on seizure threshold, mice were developed with targeted deletion of the α4 subunit (Ross et al., 2000). The behaviour of mice homozygous for this mutation was characterised in the drug-naïve state and in response to the proconvulsants pentylenetetrazole (PTZ), bicuculline, kainic acid, strychnine and 4-aminopyridine (Wong et al., 2002). Homozygous α4 Mt mice showed an enhanced seizure response with a significant excess of severe seizures and death, relative to Wt controls, in response to the GABA receptor antagonists PTZ and bicuculline. Mt mice also had an increase in kainic acid and strychnine-related minor motor seizures. Paradoxically, the seizure response to 4-aminopyridine was reduced in Mt mice. This agent causes seizures by releasing endogenous glutamate and the decreased response in Mt mice suggests that they had undergone compensatory down-regulation of glutamatergic neurotransmission.

We now report a systematic assessment of the EEG phenotype of homozygous α4 Mt mice in the drug-naïve state (during wakeful behaviour, drowsiness and sleep) and in response to the proconvulsant, PTZ.

2. Methods

2.1. Mice

Forty drug-naïve mice were studied prospectively, of which 20 were Mt and 20 were Wt. Generation of the Mt mice and their genetic background is as described previously (Ross et al., 2000). Mt mice that had been backcrossed twice to the C57BL/6 strain were used to generate 10 mating pairs of mice for interbreeding and 10 mating pairs of Wt breeders. The breeding strategy was designed to maximise heterogeneity in the genetic background of mice used in the study. Mating pairs within a genotype were interchanged frequently and randomly so as not to inadvertently select for potential modifier genes. Study mice were also selected singly from different litters to avoid assessing sibling clusters. Fifty percent of each group were male. The mean age (±standard error) at the time of injection was 126 ± 5 days in the Wt mice and 134 ± 5 days in the Mt group (not significantly different). Blinding with respect to genotype was maintained for all electrode insertions, recording sessions and scoring procedures. Blinding with respect to gender was incomplete during experimental procedures but was maintained for all EEG analyses.

2.2. Experimental procedures

All procedures were approved by the local institutional ethics committee. Under a light anaesthetic with chloral hydrate (4.0%, 0.01 ml/g), each mouse was fitted with four tungsten epidural recording electrodes in the following positions: front right and left, 1.5 mm anterior to bregma and 1.5 mm lateral to the midline; posterior right and left, 3 mm posterior to bregma and 1.5 mm lateral to the midline. Electrodes were secured to the skull with dental cement.

When mice had completely recovered from the procedure (usually 3 days later), a baseline EEG was recorded for 2–3 h. The voltage difference between the two frontal electrodes was recorded as the anterior EEG channel while the voltage difference between the two posterior electrodes was recorded as the posterior channel. Each channel was sampled at 1 KHz with high and low pass filtering at 1 and 80 Hz, respectively. Baseline EEG recordings were performed in the drug-naïve state, during the sleep phase of the rodent diurnal cycle. Concurrent video was obtained to document the behaviour of each animal. Mice were invariably awake at the beginning of each recording and then showed a gradual reduction in motor activity consistent with habituation. The mice subsequently became drowsy and fell asleep but usually awoke on several further occasions during the recording so that the video-EEG assessed a broad range of behavioural states. Sleep was identified on the basis of prolonged immobility on video with characteristic EEG patterns, including an increase in delta activity (0.75–4.0 Hz) during slow wave sleep.

After a further recovery period of three weeks, mice were injected with PTZ (80 mg/kg) subcutaneously over the right flank. Video and EEG were recorded for 10 min prior to injection, during injection and for at least 1 h after injection.

2.3. Scoring of electoencephalographic data

A panel of three experienced observers scored the 10-min pre-injection EEG and the first 40 min of the post-injection EEG. All EEG scorers were blinded to the genotype, age and gender of the mice. The EEGs were scored in a random order but, for each animal, the panel viewed every 10-s EEG sweep in chronological order. Where necessary, scorers used contextual information, such as evolutionary EEG changes over several sweeps, to guide the interpretation of abnormalities, as in standard clinical electroencephalography. The amplitude of spikes and sharp waves was measured relative to the amplitude of the pre-injection EEG. Table 1 displays the EEG scoring system used in this study, with categories listed in order of increasing ictal severity, and Fig. 1 (upper panel) illustrates typical examples of each abnormality. Although a variety of different ictal EEG features sometimes appeared within a single 10-s sweep, the EEG categories were regarded as mutually exclusive for the purposes of analysis and the panel classified each sweep according to the most severe ictal abnormality present in that sweep. Borderline cases were decided by majority vote. Individual seizures usually lasted for several sweeps and, as a result, the number of sweeps assigned to Category 3 reflected the total time spent fitting but exceeded the actual number of seizures. For each mouse, the 'time to first major seizure' (TFMS) was defined as the interval between PTZ injection and the first sweep assigned to Category 3. For four Wt mice not exhibiting a major seizure (where the delay to the first seizure was indefinite), a conservative TFMS of 20.3 min was assigned, as this was the longest TFMS observed across the whole cohort. This scoring system was based on previous experience with administering PTZ to normal and Mt mice and was designed to produce a broad correlation with behavioural scoring systems used by other investigators (Ferraro et al., 1999) and in our own laboratory (Wong et al., 2002). It was also designed to differentiate between EEG spindle episodes, spikes and more complex EEG features as these have different pharmacological susceptibilities and vary across different mouse species (Snead et al., 1999).

For each 10-s sweep, the EEG scoring panel also characterised the cerebral location of the dominant ictal abnormalities on a 5-point scale (1 = strongly anterior, 2 = moderately anterior, 3 = balanced over frontal and posterior channels, 4 = moderately posterior, and 5 = strongly posterior). In practice, most activity was recorded over a broad field, receiving a score of 3, so location scores 1 and 2 were pooled into 'anterior' and scores 3 and 4 were pooled into 'posterior' for the purposes of analysis. Finally, the background rhythm for each sweep was classified into four categories: normal, slow ($\geq$50% of activity $\leq$4 Hz), post-ictal suppression rhythm (trace 'R' in Fig. 1) or electrocerebral silence (a completely flat background or electrocardiograph artifact only). The time of death was recorded as the first occurrence of electrocerebral silence, regardless of persistent cardiac activity. Some 10-s sweeps were entirely replaced by ictal activity and on such occasions the background was left unclassified. In the present study, the background classification was used solely to subdivide sweeps assigned to Category 0A (that is, sweeps without any ictal activity) into two groups according to the presence (0A/R) or absence (0A) of the 'R' rhythm. This subdivision was undertaken in recognition of the fact that an EEG dominated by a post-ictal suppression rhythm has an entirely different character and prognostic significance to one in which normal cerebral rhythms are apparent, even though both may be free of epileptiform activity.

Mean spike counts per minute provided a secondary

Table 1
EEG scoring system

Ordinal EEG score	EEG description	EEG criteria (amplitude and duration)	Typical behavioural accompaniment
0A	Normal	No ictal abnormalities	Normal
0B	Isolated sharp wave(s)	≥1.5× baseline	Normal
1A	Multiple sharp waves, brief spindle episode	≥2× baseline and ≥1 s<5 s	Hypokinetic event
1B	Multiple sharp waves, longer spindle episode	≥2× baseline and ≥5 s	Hypokinetic event
2A	Spike±Slow wave	≥2× baseline, brief, sharp, alters background rhythm	Myoclonic jerk
2B	Multi-spike±Slow wave	≥3 spikes in the complex	Myoclonic jerk
2C	High frequency fast activity	≥1 s duration, replaces back-ground rhythm	Tonic spasm
3	Major seizure	≥5 s, repetitive spikes± slow waves obliterating background rhythm	Major clonic or tonic-clonic seizure
4	Cerebral death	Flat EEG or ECG artifact for ≥10 s	Death

measure of ictal activity. For this analysis, spike counts included all sharp transients resembling Type 2A or Type 2B events and the individual spikes of major seizures (Type 3 events). Sharp waves not accompanied by a slow wave or a change in the background (Type 0B events), spindles (Type 1A or 1B events) and bursts of low amplitude fast activity (Type 2C events) were not included.

All pretreatment, baseline EEGs, comprising at least 2 h recording for each of 40 mice, were also assessed off-line. Several mice exhibited EEG spindle events in the baseline period (Fig. 1B); these spindles were morphologically indistinguishable from PTZ-induced EEG spindles (trace 1A, Fig. 1A). Spikes, spike and slow wave complexes and major seizures (traces 2A, 2B, 2C and 3, Fig. 1) were not observed in the drug-free state and therefore the methods used for scoring the post-injection EEGs were not directly applicable. Instead, for quantitative comparisons of spindle activity between genotypes, an observer blinded to genotype reviewed the first 2 h of baseline EEG for each mouse. The number and the mean duration of individual spindles occurring in this period were recorded and compared across genotypes.

2.4. Statistical methods

To convert the ordinal, categorical EEG scale shown in Table 1 into a quantitative measure of prognostic significance, a time-weighted probability of death was calculated for each EEG feature. One of the motives for devising this score was to facilitate the quantification of EEG abnormalities in future studies of the effect of sub-lethal doses of PTZ. Using data from all 40 mice, the number of times that each EEG feature was followed by a death within 2.5 min was calculated ($N_{D2.5}$). This was then divided by the total number of times that the EEG feature was observed (N_T), to give an estimate of the probability of death within 2.5 min ($P_{D2.5}$) following observation of that EEG feature ($P_{D2.5} = N_{D2.5}/N_T$). A similar calculation was performed for death within 5, 10, 20 or 40 min, to give a range of probabilities for each EEG feature and each time interval. Deaths occurring outside the EEG-scoring period were included in this calculation. The final prognostic weight (PW) for each EEG feature was calculated by taking the mean of the time-specific probabilities of death ($PW = [P_{D2.5} + P_{D5} + P_{D10} + P_{D20} + P_{D40}]/5$). In theory, the PWs could range from 0, if no mice died following an occurrence of the relevant EEG feature, to 1, if all mice died within 2.5 min of an occurrence of that feature. In practice, based on data in the current study, the following PWs were assigned to each EEG category: 0A, 0.1154; 0B, 0.0847; 1A, 0.0775; 1B, 0.1795; 2A, 0.1957; 2B, 0.4000; 2C, 0.4620; 3, 0.4729; 0A/R, 0.2135. Using this weighting system, the EEG of each mouse was thus converted into a series of descriptive EEG labels, one for each 10-s sweep (e.g. 0B, 0B, 0B, 1A, 2A, ...) and then into a series of PWs (e.g. 0.0847, 0.0847, 0.0847, 0.0775, 0.1957, ...). For each mouse, the final prognostic score (PS) in each 5-min epoch was calculated by averaging the PWs obtained in that interval.

Mortality differences between the two groups were compared using Fisher's Exact Test. For comparisons of PSs and spike rates between genotypes, a two-way Repeated Measures Analysis of Variance (RM-ANOVA) was performed using genotype and time since injection (epoch number) as factors. The reported P-values for the RM-ANOVA relate to the effect of genotype after taking time from injection into account. Because the EEG PSs and spike rates were skewed and failed normality tests according to the Kolmogorov–Smirnov (K–S) method, the RM-ANOVA was repeated after normalisation of the data using a logarithmic transformation. Time-specific comparisons across genotypes were performed using the Mann–Whitney (MW) Rank Sum Test. The incidences of the various EEG features were expressed as proportions of the total scored post-injection time for each

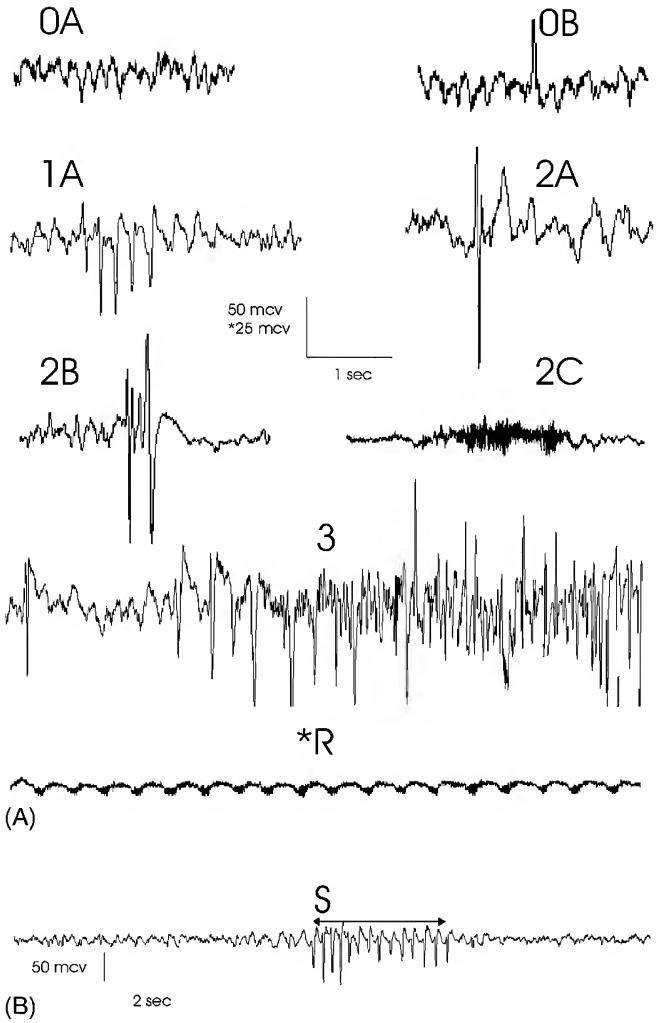

Fig. 1. (A) Representative samples of EEG following PTZ injection, including normal EEG (0A), minor sharp transient (0B), brief spindle episode (1A), spike followed by 2-s run of slow activity (2A), multi-spike and slow wave complex (2B), low amplitude fast activity (2C), onset of major seizure (3) and post-ictal suppression rhythm (R). EEG category 1B (long spindle episode) is not shown. Vertical scale: 50 mcv (*except R, 25 mcv). Horizontal scale: 1 s. (B) Anterior EEG spindle (S) occurring in a drug-naive Mt mouse, during quiet wakefulness. Vertical scale: 50 mcv. Horizontal scale: 2 s. The spindle duration, as measured between the tips of the arrowheads, was 2.49 s.

mouse and were compared across genotypes using the MW test. Where the data were normally distributed, Student's *t*-test was also performed and produced similar statistical results. For all statistical comparisons, a P value of 0.05 was interpreted as significant. Descriptive statistics are reported as mean±standard error (SE).

3. Results

3.1. Baseline EEG

Several mice (Mt mice 10, Wt mice 9) exhibited spontaneous spindles or runs of sharp waves (Fig. 1B) in the 2-h untreated, baseline period, prior to receiving any PTZ. Morphologically, these EEG features closely resembled the brief spindle episodes that were typically observed in the early phase after PTZ injection (trace 1A in Fig. 1A). The spontaneous EEG spindle events were never associated with overt behavioral arrest but frequently occurred during quiet restfulness, so a mild hypokinetic epileptic event could neither be confirmed nor excluded in any individual case. This is in contrast to the spindle events that occurred following PTZ injection that were seen to be temporarily associated with a noticeable arrest of behaviour and the animal often rested with its abdomen on the floor of the cage. We conclude that, in the absence of an interruption to normal exploratory behaviour, these spontaneous spindle events should not be considered as seizures.

The proportion of mice with spindles in the drug-free baseline period was similar in the two groups (Mt mice, 10/20, 50% vs Wt 9/20, 45%, $P = 1.0$ by Fisher's Exact Test). When spindles were observed, the spindle rate was similar in the two genotypes (Mt mice 3.8 ± 1.9 spindles/h vs Wt mice 2.2 ± 0.7 spindles/h, $P = 0.62$ by MW Rank Sum Test), as was the mean duration of individual spindles (Mt mice 1.5 ± 0.1 s vs Wt mice 1.4 ± 0.2 s, $P = 0.24$ by MW).

No major motor seizures, repetitive spikes or spike and slow wave complexes occurred prior to treatment with PTZ, regardless of genotype and state of arousal. Similarly, no fatal spontaneous seizures occurred at any time during the entire study period as no mice were found dead in their cage.

3.2. Response to pentylenetetrazole

Following PTZ administration, mortality was significantly higher in Mt mice (17/20, 85%) than Wt mice (6/20, 30%, $P < 0.001$ by Fisher's Exact Test). During the 40-min EEG scoring period, the difference in mortality was even more marked (16/20, 80% vs 3/20, 15%, $P < 0.001$), with most of the deaths occurring within 20 min of PTZ administration (15/20, 75% vs 2/20, 10%, $P < 0.001$, see Fig. 2). Amongst animals that died, the mean interval between PTZ injection and death was 20 ± 4 min in the Mt mice and 39 ± 10 min in Wt controls but this comparison was based on few Wt mice and did not achieve statistical significance ($P = 0.15$ by MW).

In contrast to this strong genotypic difference in mortality rates, there was no significant mortality difference between male and female mice (overall mortality in males 12/20, 60%; overall mortality in females 11/20, 55%, with no significant difference demonstrated by Fisher's Exact Test). Amongst Mt mice, the mortality rate amongst male and female mice was almost equal (males 8/10, 80%, females 9/10, 90%) while, amongst Wt controls, there was an excess number of deaths

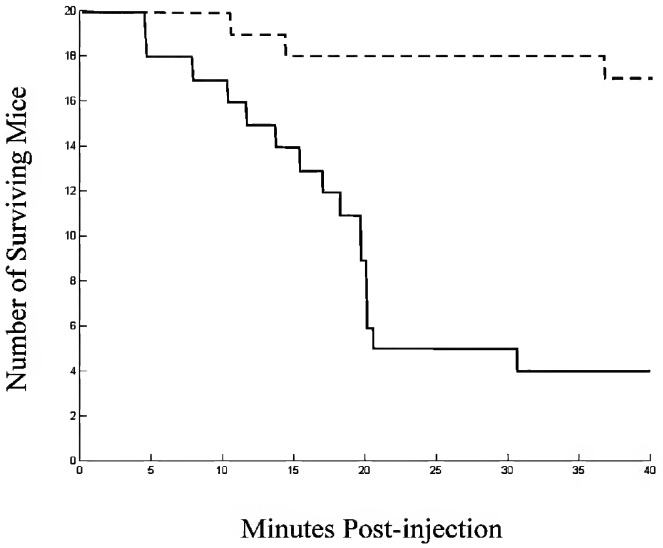

Fig. 2. Survival curve for Mt mice (solid line) and Wt mice (broken line), indicating the number of mice remaining alive at various intervals after injection of pentylenetetrazol (80 mg/kg subcutaneously). Mortality at 40 min: Mt mice 80%, Wt mice 15% ($P < 0.001$ by Fisher's Exact Test).

amongst males (4/10, 40%) compared to females (2/10, 20%); this comparison was underpowered and did not reach statistical significance.

In the 40 min that followed PTZ injection, the mean number of major seizures per mouse was significantly higher in the Mt group than the control group (Mt 3.0 ± 0.3 seizures vs Wt 2.0 ± 5, $P = 0.013$ by MW). Of the 60 major seizures that occurred in Mt mice during this period, 16 (27%) were fatal whereas, in Wt mice, only three of 39 major seizures immediately resulted in death (8%, $P = 0.020$, Fisher's Exact Test). Four Wt mice did not have a major seizure, whereas all Mt mice had at least one major seizure but this difference did not achieve statistical significance (freedom from major seizures: Mt 0/20, 0% vs Wt 4/20, 20%, $P = 0.1$ by Fisher's Exact Test). The TFMS was shorter in Mt mice than Wt mice. To allow a statistical comparison across the whole cohort, the four Wt mice that did not have a major seizure were assigned a TFMS of 20.3 min (equal to the longest observed TFMS). By this method of analysis, the genotypic difference in seizure latency was statistically significant (TFMS: Mt 4.9 ± 0.7min, vs Wt 14.3 ± 3.2min, $P = 0.035$). Amongst the 36 animals that actually had a major seizure, however, the difference in TFMS did not achieve statistical significance (TFMS: Mt 4.9 ± 0.7min, vs Wt 10.4 ± 1.9min, $P = 0.23$). In summary, the increased mortality in Mt mice reflects the fact that they were more likely to have major seizures and also that they were more likely to die during a seizure, relative to Wt controls.

The PW for each EEG feature and time interval is displayed in Fig. 3, showing that the PWs obtained for the individual EEG features were ranked in accord with expectations, justifying the ordering of the features as shown in Table 1.

In both genotypes, abnormal EEG features appeared within the first 5 min following injection, as indicated by the mean PS (Fig. 4). More severe EEG changes continued to develop over the next 15 min, particularly in Mt mice, but then declined towards pre-injection levels over the remainder of the scoring period. The EEG of Mt mice showed significantly more adverse features than those of Wt mice ($P < 0.001$ by RM-ANOVA, with or without logarithmic transformation of the scores). The difference in PSs between the groups was also statistically significant at most individual time points in the first 20 min after injection (0–5 min, $P = 0.079$; 5–10 min, $P = 0.009$; 10–15 min, $P = 0.029$; 15–20 min, $P = 0.029$, MW Rank Sum Test). It should be noted that the majority of Mt mice died within the first 20 min following injection and the mean PS for each 10-s scoring point was based exclusively on mice that were still alive at that time. Thus, some of the improvement in PS observed after 20 min reflects a selection effect. Mice that died would be expected to show more severe abnormalities than mice that survived, so the scores displayed

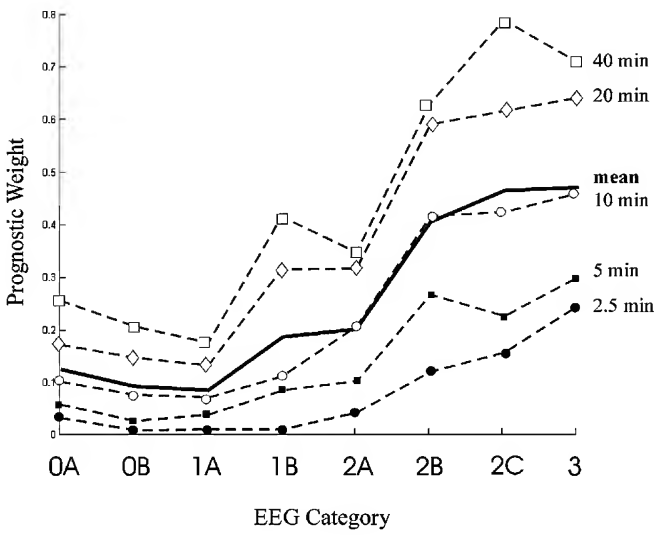

Fig. 3. Prognostic significance of individual EEG features across the entire study population ($n = 40$). For examples of each EEG feature, see Fig. 1. The number of times each EEG feature was followed by death within various time intervals (2.5, 5, 10, 20 or 40 min) is expressed as a proportion of the total number of times that the feature occurred (see text for details). Above each EEG category, the symbols indicate the increasing probabilities of death with progressively longer follow-up from an observation of that EEG feature (2.5 min, filled circles; 5 min, filled squares; 10 min, open circles; 20 min, open diamonds; 40 min, open squares). Along each broken line, from left to right, the individual points represent the increasing probability of death within the specified time interval, according to the severity of the current EEG. The solid line without symbols represents the final PW assigned to each EEG feature and equals the mean of the time-specific PWs.

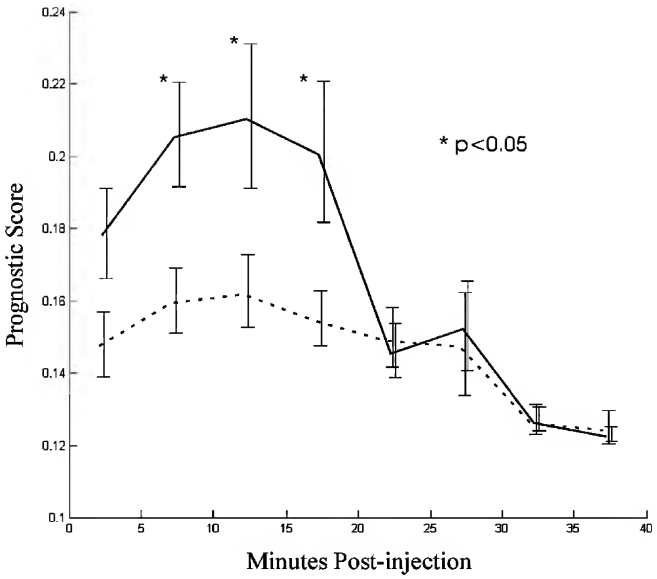

Fig. 4. Evolution of the mean (±SE) PS in Mt mice (solid line, $n = 20$) and Wt mice (broken line, $n = 20$) in the first 40 min after injection with PTZ (80 mg/kg). Higher scores indicate a predominance of adverse EEG features (see text for details). The overall difference between the two groups was statistically significant by RM-ANOVA ($P < 0.001$) and the difference was also significant throughout most individual time points in the first 20 min (0–5 min, $P = 0.079$; 5–10 min, P=0.009; 10–15 min, $P = 0.029$; 15–20 min, $P = 0.029$ by the Mann–Whitney Rank Sum Test). After 20 min, only four mice remained in the Mt group and statistical comparisons were underpowered.

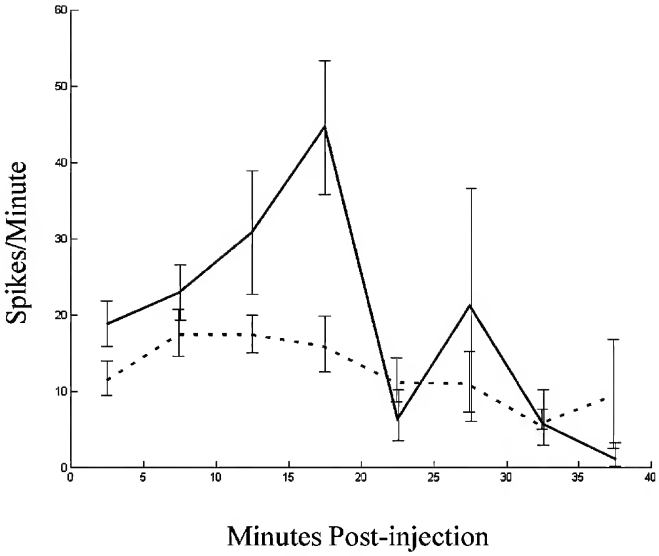

Fig. 5. The mean spike rate (±SE) in Mt mice (solid line, $n = 20$) and Wt controls (broken line, $n = 20$) in the first 40 min after subcutaneous injection with PTZ (80 mg/kg). In deriving the spike rates, the spike counts included spikes (Category 2A, see Fig. 1 and Table 1), multi-spikes (Category 2B) and the individual spikes of major seizures (Category 3). Isolated sharp transients (Category 0B), spindles (Categories 1A and 1B) and episodes of continuous fast activity (Category 2C) were not included. The difference between the genotypes was significantly different by RM-ANOVA ($P < 0.001$) for the first 20 min. After this period, only four mice remained in the Mt group and statistical comparisons were underpowered.

in Fig. 4 represent a conservative estimate of the genotypic difference in EEG abnormalities following PTZ injection.

The mean spike rate (spike counts/min) showed a similar evolution to the mean PS (Fig. 5). There was a significant excess of spiking activity in Mt mice in the first 20 min ($P < 0.001$ by RM-ANOVA, or $P = 0.041$ after logarithmic transformation of the counts). The dramatic fall in mean spike rate seen in the Mt group after 20 min reflects the death of Mt mice with high spike rates and post-ictal suppression of spiking in the few survivors. (Because only four Mt mice remained alive after 20 min, the standard error was unacceptably broad after this period and the data could not be normalised; the RM-ANOVA was therefore restricted to the first 20 min).

An analysis of the incidence of individual EEG features after PTZ injection reveals a shift of EEG phenotype in the mutants towards more severe epileptiform features (Fig. 6). There was a relative increase in the incidence of spikes in Mt mice, as reflected in the proportion of sweeps assigned to Category 2A ($P = 0.033$ by MW). There was an even more pronounced increase in multi-spike complexes (category 2B, $P = 0.002$), episodes of continuous fast activity (Category 2C, $P = 0.017$) and major seizures (Category 3, $P < 0.001$). Because the EEG categories were mutually exclusive and the incidence of each feature was expressed as a proportion of the total scored EEG time, there was a corresponding reduction in the incidence of normal EEG classifications amongst Mt mice ($P < 0.001$). Other categories, including spindles (Categories 1A and B) and isolated sharp waves (Category 0B), occurred with similar frequencies in the two groups.

An analysis of the cerebral location of the ictal abnormalities showed that most epileptiform features were recorded over the entire head. Spikes and major seizures were rarely dominant in a single channel, making it impossible to assess the effects of the mutation on specific brain regions. Thus, while a mean of 40 ± 4% of the EEG sweeps recorded from Mt mice and 19 ± 3% of sweeps recorded from Wt mice exhibited high-grade epileptiform abnormalities (categories 2A, 2B, 2C or 3), most of this activity was balanced across both EEG channels. Mt mice had high-grade epileptiform activity in both channels for a mean of 32 ± 4% of the time (79% of total high-grade activity), while Wt mice had similar activity in both channels for 14 ± 2% of the time (73% of total high-grade activity). Anterior high-grade activity was seen in Mt mice only 4.4 ± 1.2% of the time and, in Wt mice, 3.5 ± 1.0% of the time. Posterior high-grade activity was seen in Mt and Wt mice only 4.0 ± 1.2% and 1.5 ± 0.4% of the time, respectively. None of the

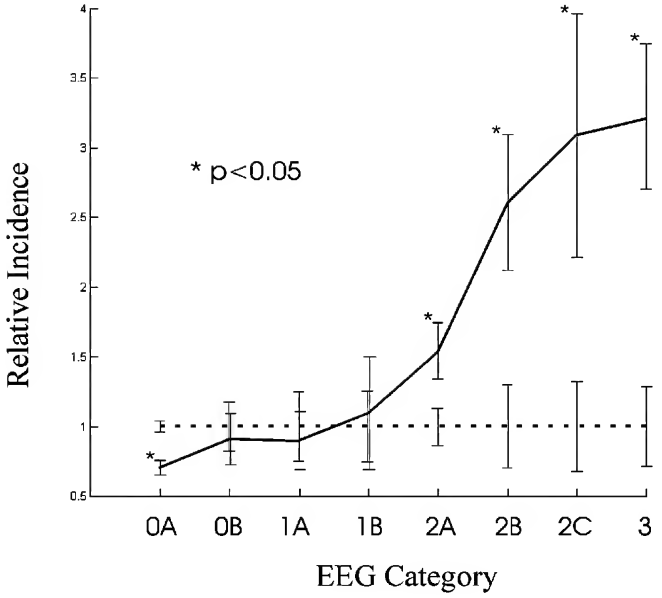

Fig. 6. The mean (±SE) relative incidence of defined EEG events in Mt mice (solid line, $n = 20$) and Wt mice (broken line, $n = 20$) in the first 40 min following injection of PTZ (80 mg/kg). Each 10-s EEG sweep from each mouse was classified according to the most severe ictal abnormality present and EEG categories are as defined in Table 1 and Fig. 1 (see text for details). The incidence of each EEG feature for each mouse was expressed as a proportion of the total EEG time and divided by the mean incidence of the feature in Wt controls. There was a significant excess of spikes (2A, $P = 0.033$ by Mann–Whitney Rank Sum Test), multi-spikes (2B, $P = 0.002$), continuous fast activity (2C, $P = 0.017$) and major seizures (3, $P < 0.001$) in Mt mice, relative to Wt controls.

regional differences in high-grade activity approached statistical significance. Low-grade epileptiform activity (categories 0B, 1A and 1B) was seen in Mt mice and Wt mice 8.1 ± 1.5% and 8.9 ± 1.5% of the time, respectively, and also failed to show significant differences in regional distribution between the two genotypes.

4. Discussion

PTZ causes seizures by antagonising $GABA_A$ receptors (Snead et al., 1999). It binds to a site related to but distinct from the picrotoxin binding site and single channel recordings suggest that, once bound, PTZ decreases the probability of chloride channel opening without affecting open channel conduction or duration of channel opening (Huang et al., 2001).

As a proconvulsant, PTZ has been used in low-dose (10–20 mg/kg) and high-dose (50–80 mg/kg) seizure models (Maresceaux et al., 1984; Snead et al., 1999). At low doses in rats, PTZ produces an electroclinical picture that resembles absence epilepsy, with regular spike and wave complexes and behavioural arrest, sometimes accompanied by minor myoclonic activity of the vibrissae (Maresceaux et al., 1984; Snead et al., 1999). In mice, low doses have been associated with brief spindle episodes that are usually accompanied by behavioural arrest without myoclonic activity (Snead et al., 1999). In both species, these low-dose, non-convulsive PTZ phenomena are not associated with post-ictal EEG slowing, a feature they share with absence episodes in humans. They also have a pharmacological response profile which resembles that of human absence episodes, in that they are prevented by drugs such as sodium valproate but are not diminished by drugs such as carbamazepine (Maresceaux et al., 1984).

At higher doses (50–80 mg/kg), PTZ produces focal myoclonus and generalised clonic or clonic-tonic seizures (Ferraro et al., 1999; Snead et al., 1999). The EEG typically shows spikes, spike and slow wave complexes and, during major seizures, continuous spike and slow wave activity with significant post-ictal slowing. Compared to the spindle episodes associated with low-dose PTZ, these high-grade EEG abnormalities have a completely different pharmacological profile in that they are sensitive to carbamazepine (De Feo et al., 1991; Snead et al., 1999). Because uncontrolled observations suggest that human ADNFLE is particularly responsive to carbamazepine (Picard et al., 1999, 2000; Berkovic and Scheffer, 2002), the convulsive phenomena observed with high-dose PTZ potentially provide a better model for ADNFLE than the milder absence-like events occurring with low-dose PTZ. These considerations, along with prior observations that α4 Mt mice are susceptible to high-dose PTZ (Wong et al., 2002), motivated the choice of proconvulsant agent and the dose (80 mg/kg) in the current study.

The results presented here confirm previous observations (Wong et al., 2002) that lack of effective α4 nAChR subunits renders mice highly susceptible to high-dose PTZ. This heightened susceptibility was reflected in all endpoints studied. The overall mortality rate was higher in Mt mice, as was early mortality within the first 20 or 40 min post-injection. This does not simply reflect that Mt mice had an increased mortality rate for a given seizure type, because a quantitative analysis of observer-rated EEG abnormalities showed a sustained elevation in adverse prognostic features in the Mt mice. These abnormalities appeared within the first 5 min after injection, rose to a peak within 10–20 min and returned towards baseline over the remaining 25 min. A genotypic difference in the mean 5-min EEG PSs was readily apparent throughout the first 20 min after injection and was statistically significant from 5–15 min. Following this, only a small minority of Mt mice remained alive so comparisons between genotypes were unreliable. Mean spike counts per minute showed a similar evolution over the first 40 min post-injection.

An analysis of the incidence of individual EEG features shows that it was the more severe epileptiform abnormalities, known to be associated with high-dose

PTZ, that were more prevalent in Mt mice. In particular, multi-spikes, multi-spike and slow wave complexes and major seizures were all significantly more frequent in the Mt mice. Spindle-like episodes, similar to those previously associated with low-dose PTZ, were typically observed in the first few minutes following PTZ injection or during the recovery phase but were no more prevalent in the Mt mice than in Wt controls. Because the pharmacological response-profile of ADNFLE (i.e. carbamazepine responsiveness) resembles that of *convulsive*, high-dose PTZ phenomena but differs from *non-convulsive*, low-dose PTZ phenomena, the finding that absence of the α4/β2 receptor complex predisposes mice to high-grade abnormalities, but not to spindles, is of interest. It is consistent with the idea that seizures following high-dose PTZ might share some mechanisms with the spontaneous seizures occurring in ADNFLE, whereas the hypokinetic absence-like episodes and EEG spindles are likely to result from distinct mechanisms not involving the α4/β2 nAChR.

The failure to observe spontaneous seizures in the α4 Mt mice, as reported in the present study, means that these mice do not provide a faithful model of all aspects of ADNFLE. Sleep regulation in mice may not be sufficiently similar to sleep regulation in humans to produce a spontaneous seizure phenotype in mice resembling ADNFLE. It is also possible that prolonged monitoring may be required to identify infrequent spontaneous seizures in Mt mice. ADNFLE has incomplete penetrance in humans (approximately 65–75%, Tassinari and Michelucci, 1997; Berkovic and Scheffer, 2002). Furthermore, seizures in ADNFLE generally do not occur until humans are more than 5 years old and are often delayed until after 10 years (Tassinari and Michelucci, 1997; Berkovic and Scheffer, 2002). Thus, spontaneous seizures might only occur when α4 nAChR hypofunction occurs in the appropriate genetic background and at a specific stage of development. Crossbreeding experiments are now underway to test this hypothesis and to assess the seizure threshold of heterozygote Mt mice.

The exaggerated electrographic response of α4 Mt mice to PTZ, as demonstrated in the present study, parallels previous observations of the behavioural response of α4 Mt mice to proconvulsants including PTZ (Wong et al., 2002). In the earlier study, a higher proportion of Mt mice exhibited major seizures in response to PTZ 80 mg/kg than did controls and the mortality was also significantly higher in the Mt mice. Similar findings were obtained with a different GABA antagonist, bicuculline, and with the glycine antagonist, strychnine. The α4 nAChR subunit, therefore, provides partial protection against a range of proconvulsants. The known neuromodulatory role of α4/β2 nAChRs and previous demonstrations that nicotinic agonists facilitate release of GABA in most brain regions including the neocortex (Lu et al., 1998), provide a plausible basis for this protective effect. GABAergic cortical interneurons bearing post-synaptic α4/β2 nAChRs may also be involved (Porter et al., 1999). Furthermore, a deficit in dopaminergic function might contribute to the enhanced susceptibility of Mt mice to proconvulsants, given that nicotinic agonists are known to facilitate dopamine release (Grady et al., 2001; Wonnacott, 1997) and dopamine antagonism with antipsychotics in humans lowers the seizure threshold (Arana, 2000). Consistent with this hypothesis, studies in our laboratory have shown architectural changes in the axonal arborisation of nigrostriatal neurons of Mt mice, suggesting a disturbance of dopaminergic function (data not shown).

In ADNFLE, disturbed nicotinic receptor function results in spontaneous seizures, a situation that clearly differs from the present model, where the protective role of the nicotinic receptors has been unmasked with a proconvulsant. This study confirms, however, that functioning α4 nAChRs provide partial protection against the proconvulsant-effects of the GABA antagonist, PTZ, in vivo. Given the conflicting evidence regarding the functional effect of ADNFLE mutations in vitro, these results provide useful additional support for the hypothesis that ADNFLE may be caused by α4 nAChR hypofunction.

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